

IMPROVED METHOD FOR PREPARING FLOUR DOUGHS AND PRODUCTS
MADE FROM SUCH DOUGHS USING GLYCEROL OXIDASE

FIELD OF THE INVENTION

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The present invention relates to the field of food manufacturing, in particular to the preparation of improved bakery products and other farinaceous food products. Specifically, the invention concerns the use of glycerol oxidase as a dough 10 strengthening agent and improvement of the quality of baked and dried products made from such improved doughs. There is also provided a method of improving the properties of doughs and baked product by combined use of glycerol oxidase and a lipase.

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TECHNICAL BACKGROUND AND PRIOR ART

The "strength" or "weakness" of doughs is an important aspect 20 of making farinaceous finished products from doughs, including baking. The "strength" or "weakness" of a dough is primarily determined by its content of protein and in particular the content and the quality of the gluten protein is an important factor in that respect. Flours with a low protein 25 content is generally characterized as "weak". Thus, the cohesive, extensible, rubbery mass which is formed by mixing water and weak flour will usually be highly extensible when subjected to stress, but it will not return to its original dimensions when the stress is removed.

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Flours with a high protein content are generally characterized as "strong" flours and the mass formed by mixing such a flour and water will be less extensible than the mass formed from a weak flour, and stress which is applied during mixing 35 will be restored without breakdown to a greater extent than is the case with a dough mass formed from a weak flour. Strong flour is generally preferred in most baking contexts because of the superior rheological and handling properties

of the dough and the superior form and texture qualities of the finished baked or dried products made from the strong flour dough.

5 Doughs made from strong flours are generally more stable. Stability of a dough is one of the most important characteristics of flour doughs. Within the bakery and milling industries it is known to use dough "conditioners" to strengthen the dough to increase its stability and strength. Such 10 dough conditioners are normally non-specific oxidizing agents such as e.g. iodates, peroxides, ascorbic acid, K-bromate or azodicarbonamide and they are added to dough with the aims of improving the baking performance of flour to achieve a dough with improved stretchability and thus having a desirable 15 strength and stability. The mechanism behind this effect of oxidizing agents is that the flour proteins, in particular gluten contains thiol groups which, when they become oxidized, form disulphide bonds whereby the protein forms a more stable matrix resulting in a better dough quality and improvements 20 of the volume and crumb structure of the baked products.

However, the use of several of the currently available non-specific oxidizing agents is either objected to by consumers 25 or is not permitted by regulatory bodies. Hence it has been attempted to find alternatives to these conventional flour and dough additives, and the prior art has i.a. suggested the use of glucose oxidase and hexose oxidase for this purpose.

30 Glycerol oxidase is an oxidoreductase which is capable of oxidizing glycerol. Different types of glycerol oxidase have been described in the literature. Some of these glycerol oxidases need co-factors in order to oxidize glycerol (Shuen-Fu et al., 1996. Enzyme Micro. Technol., 18:383-387).

35 However, glycerol oxidase from *Aspergillus japonicus* does not require any co-factors in the oxidation of glycerol to glyceraldehyde (T. Uwajima and O. Terada, 1980. Agri. Biol.

Chem. 44:2039-2045).

This glycerol oxidase has been characterized by T. Uwajima and O. Terada (Methods in Enzymology, 1982, 89:243-248) and 5 T. Uwajima et al. (Agric. Biol. Chem., 1979, 43:2633-2634), and has a pH optimum at 7.0 and K_m and V_{max} are 10.4 mM and 935.6 $\mu\text{mol H}_2\text{O}_2 \text{ min}^{-1}$ respectively using glycerol as substrate. The enzyme is most active on glycerol but also other substrates like dihydroxyacetone, 1,3-propanediol, D-galactose and D-fructose are oxidized by glycerol oxidase.

Glycerol oxidase not requiring co-factors has also been isolated from *Penicillium* and characterized by Shuen-Fuh Lin et al. (Enzyme Micro. Technol., 1996, 18:383-387). This 15 enzyme has optimum activity in the pH range from 5.5 to 6.5 at 30°C. The enzyme is stable between 20 and 40°C but loses its activity at temperatures above 50°C.

Other potential sources for glycerol oxidase according to the 20 invention include different fungal species as disclosed in DE-2817087-A, such as *Aspergillus oryzae*, *Aspergillus parasiticus*, *Aspergillus flavus*, *Neurospora crassa*, *Neurospora sitophila*, *Neurospora tetrasperma*, *Penicillium nigriceps*, *Penicillium funiculosum* and *Penicillium janthinellum*.

Glycerol oxidase isolated from the above natural sources has 25 been used for different applications. Thus, glycerol oxidase from *Aspergillus japonicus* has been used for glycoaldehyde production from ethylene glycol (Kimiya Isobe and Hiroshi Nishise, 1995, Journal of Molecular Catalysis B: Enzymatic, 1:37-43). Glycerol oxidase has also been used in the combination with lipoprotein lipase for the determination of 30 contaminated yolk in egg white (Yoshinori Mie, 1996. Food Research International, 29:81-84). DE-2817087-A and US 35 4,399,218 disclose the use of glycerol oxidase for the determination of glycerol.

It has now been found that the addition of a glycerol oxidase to a flour dough results in an increased resistance hereof to deformation when the dough is stretched, i.e. this enzyme confers to the dough an increased strength whereby the dough 5 becomes less prone to mechanical deformation. Accordingly, glycerol oxidase is highly useful as a dough conditioning agent in the manufacturing of flour dough based products including not only bread products but also other products made from flour doughs such as noodles and alimentary paste 10 products.

It has also been found that the dough strengthening effect of glycerol oxidase is potentiated significantly when it is combined with a lipase, which in itself does not affect the 15 dough strength. Furthermore, the combined use of glycerol oxidase and lipase results in an improvement of bread quality, in particular in respect of specific volume and crumb homogeneity, which is not a simple additive effect, but reflects a synergistic effect of these two types of enzymes.

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SUMMARY OF THE INVENTION

Accordingly, the invention relates in a first aspect to a 25 method of improving the rheological properties of a flour dough and the quality of the finished product made from the dough, comprising adding to the dough 10 to 10,000 units of a glycerol oxidase per kg of flour.

30 In a further aspect there is provided a method of improving the rheological properties of a flour dough and the quality of the finished product made from the dough, comprising adding to the dough a glycerol oxidase and a lipase.

35 The invention pertains in a still further aspect to dough improving composition comprising a glycerol oxidase and at least one further dough ingredient or dough additive.

In still further aspects, the invention relates to the use of a glycerol oxidase for improving the rheological properties of a flour dough and the quality of the finished product made from the dough and to the use of a glycerol oxidase and a 5 lipase in combination for improving the rheological properties of a flour dough and the quality of the finished product made from the dough.

10 DETAILED DISCLOSURE OF THE INVENTION

In one aspect, the present method provides a method of improving the rheological properties of flour doughs.

15 The expression "rheological properties" as used herein refers particularly to the effects of dough conditioners on dough strength and stability as the most important characteristics of flour doughs. According to American Association of Cereal Chemists (AACC) Method 36-01A the term "stability" can be 20 defined as "the range of dough time over which a positive response is obtained and that property of a rounded dough by which it resists flattening under its own weight over a course of time". According to the same method, the term "response" is defined as "the reaction of dough to a known 25 and specific stimulus, substance or set of conditions, usually determined by baking it in comparison with a control"

As it is mentioned above, it is generally desirable to improve the baking performance of flour to achieve a dough with 30 improved stretchability and thus having a desirable strength and stability by adding oxidizing agents which cause the formation of protein disulphide bonds whereby the protein forms a more stable matrix resulting in a better dough quality and improvements of the volume and crumb structure of 35 baked products.

Thus, the term "rheological properties" relates to the above physical and chemical phenomena which in combination will

determine the performance of flour doughs and thereby also the quality of the resulting products.

The method comprises, as it is mentioned above, the addition 5 of an effective amount of a glycerol oxidase to the dough. It will be understood that the addition can be either to a component of the dough recipe or to the dough resulting from mixing all of the components for the dough. In the present context, "an effective amount" is used to indicate that the 10 amount is sufficient to confer to the dough and/or the finished product improved characteristics as defined herein. Specifically, such an amount is in the range of 10 to 10,000 units of glycerol oxidase per kg flour.

15 In one useful embodiment of the method according to the invention, the glycerol oxidase can, as it is described in details herein, be isolated from a bacterial species, a fungal species, a yeast species, an animal cell including a human cell or a plant cell. Examples of glycerol oxidase 20 producing fungal species are species belonging to the genera *Aspergillus*, *Neurospora* and *Penicillium*, such as *A. japonicus*, *A. oryzae*, *A. parasiticus*, *A. flavus*, *Neurospora crassa*, *N. sitophila*, *N. tetrasperma*, *Penicillium nigricans*, *P. funiculosum* and *P. janthinellum*.

25 Glycerol oxidase can be derived as a native enzyme from natural sources such as the above.

It is one objective of the invention to provide improved 30 bakery products. In accordance with the invention, a bakery product dough including a bread dough is prepared by mixing flour with water, a leavening agent such as yeast or a conventional chemical leavening agent, and an effective amount of glycerol oxidase under dough forming conditions. It is, 35 however, within the scope of the invention that further components can be added to the dough mixture.

Typically, such further dough components include conventional

nally used dough components such as salt, sweetening agents such as sugars, syrups or artificial sweetening agents, lipid substances including shortening, margarine, butter or an animal or vegetable oil, glycerol and one or more dough 5 additives such as emulsifying agents, starch degrading enzymes, cellulose or hemicellulose degrading enzymes, proteases, lipases, non-specific oxidizing agents such as those mentioned above, flavouring agents, lactic acid bacterial cultures, vitamins, minerals, hydrocolloids such as alginates, 10 carrageenans, pectins, vegetable gums including e.g. guar gum and locust bean gum, and dietary fiber substances.

Conventional emulsifying agents used in making flour dough products include as examples monoglycerides, diacetyl tar-
15 taric acid esters of mono- and diglycerides of fatty acids, and lecithins e.g. obtained from soya. Among starch degrading enzymes, amylases are particularly useful as dough improving additives. Other useful starch degrading enzymes which may be added to a dough composition include glucoamylases and pullu-
20 lanases. In the present context, further interesting enzymes are xylanases and oxidoreductases such as glucose oxidase, pyranose oxidase, hexose oxidase, sulphhydryl oxidase, and lipases.

25 A preferred flour is wheat flour, but doughs comprising flour derived from other cereal species such as from rice, maize, barley, rye and durra are also contemplated.

In accordance with the invention, the dough is prepared by admixing flour, water, the glycerol oxidase and optionally other ingredients and additives. The glycerol oxidase can be added together with any dough ingredient including the water or dough ingredient mixture or with any additive or additive mixture. The dough can be prepared by any conventional dough preparation method common in the baking industry or in any other industry making flour dough based products.

The glycerol oxidase can be added as a liquid preparation or in the form of a dry powder composition either comprising the enzyme as the sole active component or in admixture with one or more other dough ingredients or additive.

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The amount of the glycerol oxidase added is an amount which results in the presence in the dough of 10 to 5,000 units (as defined in the following) such as 10 to 2,500 units per kg of flour. In useful embodiments, the amount is in the range of 10 20 to 1,500 units per kg of flour.

The effect of the glycerol oxidase on the rheological properties of the dough can be measured by standard methods according to the International Association of Cereal Chemistry (ICC) and the American Association of Cereal Chemistry (AACC) including the amylograph method (ICC 126), the farinograph method (AACC 54-21) and the extensigraph method (AACC 54-10). The AACC method 54-10 defines the extensigraph in the following manner: "the extensigraph records a load-extension 20 curve for a test piece of dough until it breaks. Characteristics of load-extension curves or extensigrams are used to assess general quality of flour and its responses to improving agents". In effect, the extensigraph method measures the relative strength of a dough. A strong dough exhibits a 25 higher and, in some cases, a longer extensigraph curve than does a weak dough.

In a preferred embodiment of the method according to the invention, the resistance to extension of the dough in terms 30 of the ratio between the resistance to extension (height of curve, B) and the extensibility (length of curve, C), i.e. the B/C ratio as measured by the AACC method 54-10 is increased by at least 10% relative to that of an otherwise similar dough not containing glycerol oxidase. In more preferred embodiments, the resistance to extension is increased 35 by at least 20%, such as at least 50% and in particular by at least 100%.

It has been found that the addition of glycerol oxidase to bakery product doughs results in bakery products such as yeast leavened and chemically leavened products in which the 5 specific volume is increased relative to an otherwise similar bakery product, prepared from a dough not containing glycerol oxidase. In this context, the expression "specific volume" is used to indicate the ratio between volume and weight of the product. It has been found that, in accordance with the above 10 method, the specific volume can be increased significantly such as by at least 10%, preferably by at least 20%, including by at least 30%, preferably by at least 40% and more preferably by at least 50%.

15 The method according to the invention is highly suitable for improving the rheological properties and quality of the finished products of conventional types of yeast leavened bread products based on wheat flour, such as loaves and rolls. The method is also suitable for improving the rheo- 20 logical properties of doughs containing chemical leavening agents (baking powder) and the quality of products made from such doughs. Such products include as examples sponge cakes and muffins.

25 In one interesting aspect, the invention is used to improve the rheological properties of doughs intended for noodle products including "white noodles" and "chinese noodles" and to improve the textural qualities of the finished noodle products. A typical basic recipe for the manufacturing of 30 noodles comprises the following ingredients: wheat flour 100 parts, salt 0.5 parts and water 33 parts. Furthermore, glycerol is often added to the noodle dough. The noodles are typically prepared by mixing the ingredients in an appropriate mixing apparatus followed by rolling out the noodle dough 35 using an appropriate noodle machine to form the noodle strings which are subsequently air dried.

The quality of the finished noodles is assessed i.a. by their

colour, cooking quality and texture. The noodles should cook as quickly as possible, remain firm after cooking and should preferably not loose any solids to the cooking water. On serving the noodles should preferably have a smooth and firm surface not showing stickiness and provide a firm "bite" and a good mouthfeel. Furthermore, it is important that the white noodles have a light colour.

Since the appropriateness of wheat flour for providing
10 noodles having the desired textural and eating qualities may
vary according to the year and the growth area, it is usual
to add noodle improvers to the dough in order to compensate
for sub-optimal quality of the flour. Typically, such
improvers will comprise dietary fiber substances, vegetable
15 proteins, emulsifiers and hydrocolloids such as e.g. al-
ginates, carrageenans, pectins, vegetable gums including guar
gum and locust bean gum, and amylases, and as mentioned
above, glycerol.

20 It is therefore an important aspect of the invention that the glycerol oxidase according to the invention is useful as a noodle improving agent optionally in combination with glycerol and other components currently used to improve the quality of noodles. Thus, it is contemplated that noodles
25 prepared in accordance with the above method will have improved properties with respect to colour, cooking and eating qualities including a firm, elastic and non-sticky texture and consistency.

30 In a further useful embodiment, the dough which is prepared by the method according to the invention is a dough for preparing an alimentary paste product. Such products which include as examples spaghetti and maccaroni are typically prepared from a dough comprising main ingredients such as 35 flour, eggs or egg powder and/or water. After mixing of the ingredient, the dough is formed to the desired type of paste product and air dried. It is contemplated that the addition of glycerol oxidase to a paste dough, optionally in combina-

tion with glycerol, will have a significant improving effect on the extensibility and stability hereof resulting in finished paste product having improved textural and eating qualities.

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In a useful embodiment, there is provided a dough improving method wherein at least one further enzyme is added to the dough ingredient, dough additive or the dough. In the present context, suitable enzymes include cellulases, hemicellulases, 10 xylanases, starch degrading enzymes, oxidoreductases and proteases.

In a further aspect, the invention relates to a method of improving the rheological properties of a flour dough and the 15 quality of the finished products made from the dough which comprises that both a glycerol oxidase and a lipase is added to the dough.

It was surprisingly found that the two types of enzymes were 20 capable of interacting with each other under the dough conditions to an extent where the effect on improvement of the dough strength and bread quality by the enzymes was not only additive, but the effect was synergistic.

25 Thus, with respect to improvement of dough strength it was found that with glycerol oxidase alone, the B/C ratio as measured after 45 minutes of resting was increased by 34%, with lipase alone no effect was observed. However, when combining the two enzymes, the B/C ratio was increased by 30 54%, i.e. combining the glycerol oxidase with the lipase enhanced the dough strengthening effect of glycerol oxidase by more than 50%. Thus, one objective of combining glycerol oxidase and a lipase is to provide an enhancement of the dough strengthening effect of glycerol oxidase by at least 35 25% such as at least 50% including at least 75%, determined as described herein.

In relation to improvement of finished product, it was found

that the combined addition of glycerol oxidase and a lipase resulted in a substantial synergistic effect in respect to crumb homogeneity as defined herein. Also, with respect to the specific volume of baked product a synergistic effect was 5 found. Thus, for a bread product, the addition of lipase alone typically results in a negligible increase of the specific volume, addition of glycerol oxidase alone in an increase of about 25%, whereas a combined addition of the two enzymes results in an increase of more than 30%.

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Further in relation to improvement of the finished product, it was found that the addition of lipase resulted in modification of the glycolipids, monogalactosyl diglyceride and digalactosyl diglyceride present in dough. These components 15 were converted to the more polar components monogalactosyl monoglyceride and digalactosyl monoglyceride. As galactosyl monoglycerides are more surface active components than galactosyl diglycerides it is assumed that galactosyl monoglycerides contributed to the observed improved crumb cell structure and homogeneity. Thus, one objective of using lipase is 20 to hydolyse at least 10% of the galactosyl diglycerides normally present in a flour dough to the corresponding galactosyl monoglycerides, such as at least 50% including at least 100%.

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The details of such a method using combined addition of glycerol oxidase and lipase are, apart from the use of a lipase in combination with glycerol oxidase, substantially similar to those described above for a method according to 30 the invention which does not require the addition of a lipase.

When using, in accordance with the invention, a lipase in combination with a glycerol oxidase, the amount of lipase is 35 typically in the range of 10 to 100,000 lipase units (LUS) (as defined in the following) per kg flour including the range of 10 to 20,000 LUS, e.g. 100 to 15,000 LUS such as 500 to 10,000 LUS.

Lipases that are useful in the present invention can be derived from a bacterial species, a fungal species, a yeast species, an animal cell and a plant cell. Whereas the enzyme 5 may be provided by cultivating cultures of such source organisms naturally producing lipase, it may be more convenient and cost-effective to produce it by means of genetically modified cells such as it is described in details in the following examples. In the latter case, the term "derived" 10 may imply that a gene coding for the lipase is isolated from a source organism and inserted into a host cell capable of expressing the gene.

Thus, the enzyme may in a useful embodiment be derived from 15 an *Aspergillus* species including as examples *A. tubigensis*, *A. oryzae* and *A. niger*.

Presently preferred lipases include the lipase designated Lipase 3, the production and characteristics of which is 20 described in details in the following examples, or a mutant of this enzyme. In the present context, the term "mutant" refers to a lipase having, relative to the wild-type enzyme, an altered amino acid sequence. A further preferred lipase is the lipase found in the commercial product, GRINDAMYLTM EXEL 25 16.

In a further aspect of the invention there is provided a 30 dough improving composition comprising a glycerol oxidase and at least one further dough ingredient or dough additive.

The further ingredient or additive can be any of the ingredients or additives which are described above. The composition may conveniently be a liquid preparation comprising the 35 glycerol oxidase. However, the composition is conveniently in the form of a dry composition.

The amount of the glycerol oxidase in the composition is in the range of 10 to 10,000 units per kg flour. It will be

appreciated that this indication of the amount of enzyme implies that a recommended appropriate amount of the composition will result in the above stated amount in the dough to which it is added. In specific embodiments, the amount of 5 glycerol oxidase is in the range of 10 to 5,000 units such as 10 to 2,500 units per kg of flour. In other useful embodiments, the amount is in the range of 20 to 1,500 units per kg of flour.

10 In another embodiment, the dough improving composition may further comprises a lipase as defined above and in the amounts as also described above in relation to the method according to the invention.

15 Optionally, the composition is in the form of a complete dough additive mixture or pre-mixture for making a particular finished product and containing all of the dry ingredients and additives for such a dough. In specific embodiments, the composition is one particularly useful for preparing a baking 20 product or in the making of a noodle product or an alimentary paste product.

In one advantageous embodiment of the above method at least one further enzyme is added to the dough. Suitable examples 25 hereof include a cellulase, a hemicellulase, a xylanase, a starch degrading enzyme, hexose oxidase and a protease.

In a preferred advantageous embodiment, the further added enzyme is a lipase. It has been found that in accordance with 30 the above method, the crumb homogeneity and specific volume of the bakery product can be increased significantly as compared to that of an otherwise similar bakery product prepared from a dough not containing glycerol oxidase, and from a similar bakery product prepared from a dough containing 35 glycerol oxidase.

In a still further aspect, the present invention pertains to the use of a glycerol oxidase and a lipase in combination for

improving the rheological properties of a flour dough and the quality of the finished product made from the dough.

In this connection, specific embodiments include use wherein 5 the improvement of the rheological properties of the dough include that the resistance to extension of the dough in terms of the ratio between resistance to extension (height of curve, B) and the extensibility (length of curve, C), i.e. the B/C ratio, as measured by the AACC method 54-10 is 10 increased by at least 10% relative to that of an otherwise similar dough that does not contain glycerol oxidase and use wherein the improvement of the quality of the finished product made from the dough is that the average pore diameter of the crumb of the bread made from the dough is reduced by at 15 least 10%, relative to a bread which is made from a bread dough without addition of the lipase.

In a further embodiment, the use according to the invention, implies that the improvement of the quality of the finished 20 product made from the dough consists in that the pore homogeneity of the crumb of the bread made from the dough is increased by at least 5%, relative to a bread which is made from a bread dough without addition of the lipase. The pore homogeneity of bread is conveniently measured by means of an 25 image analyzer composed of a standard CCD-video camera, a video digitiser and a personal computer with WinGrain software. Using such an analyzer, the results of pore diameter in mm and pore homogeneity can be calculated as an average of measurements from 10 slices of bread. The pore homogeneity is 30 expressed in % of pores that are larger than 0.5 times the average of pore diameter and smaller than 2 times the average diameter.

In a further embodiment, the use relates to improvement of 35 the rheological characteristics of the dough including that the gluten index (as defined hereinbelow) in the dough is increased by at least 5%, relative to a dough without addition of a lipase, the gluten index is determined by means

of a Glutomatic 2200 apparatus.

BRIEF DESCRIPTION OF THE FIGURES

5 The present invention is further illustrated by reference to the accompanying figures in which

Fig. 1 shows the restriction map of the genomic clone of the 10 *lipA* gene,

Fig. 2 shows the structure of the *lipA* gene encoding lipase 3,

Fig. 3 shows a chromatogram of HIC fractionated culture 15 supernatant of an *Aspergillus tubigensis* transformant with 62-fold increase of lipase 3, and

Fig. 4 shows a chromatogram of HIC fractionated culture 20 supernatant of the untransformed *Aspergillus tubigensis* strain.

The invention will now be described by way of illustration in the following non-limiting examples.

25 A. PRODUCTION AND PURIFICATION OF GLYCEROL OXIDASE (GLOX)

EXAMPLE 1

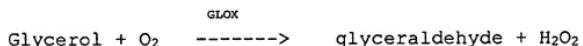
Production, extraction and purification of glycerol oxidase
30 using different strains and cultivation conditions

1. Production, extraction and purification of glycerol
oxidase using *Aspergillus japonicus* ATCC 1042 cultivated in a
production medium containing 3% glycerol

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The following assay for determination of glycerol oxidase activity was used:

The assay is based on the method described by Sullivan and Ikawa (Biochimica and Biophysica Acta, 1973, 309:11-22), but modified as described in the following. An assay mixture containing 150 μ l 2% glycerol (in 100 mM phosphate buffer, pH 5 7.0), 120 μ l 100 mM phosphate buffer, pH 7.0, 10 μ l o-dianisidin dihydrochloride (Sigma D 3252, 3 mg/ml in H₂O), 10 μ l peroxidase (POD) (Sigma P8125, 0.1 mg/ml in 100 mM phosphate buffer, pH 7.0) and 10 μ l glycerol oxidase (GLOX) solution. The controls are made by adding buffer in place of 10 GLOX solution. The incubation is started by the addition of 15 glycerol. After 15 minutes of incubation at 25°C in microtiter plates, the absorbance at 402 nm is read in a Elisa reader. A standard curve is constructed using varying concentrations of H₂O₂ in place of the enzyme solution. The reaction can be described in the following manner:



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Oxidised o-dianisidine has a yellow colour absorbing at 402 25 nm.

One glycerol oxidase unit (U) is the amount of enzyme which catalyses the production of 1 μ mole H₂O₂ per minute at 25°C, pH 7.0 at a substrate concentration of 0.2 M glycerol.

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A spore suspension of *Aspergillus japonicus* ATCC 1042 was prepared by incubating *A. japonicus* on PDA medium (30°C, 7 days) and washing with 10 ml of 0.2% Tween 80. A preculture was prepared by inoculating 1 ml of the resulting spore 35 suspension in 300 ml production medium containing 3.0 % of glycerol (87%, Merck), 0.3% of yeast extract (Difco), 0.1% of meat extract (Difco), 0.1 % KH₂PO₄ (Merck), 0.1% of MGSO₄ *

7H₂O (Merck), 0.1% antifoam (Contra spum) and 70 mg/l of chloramphenicol (Mecobenzon) (pH adjusted to 7.2 with NaOH) in a 500 ml flask. The preculture was incubated overnight at 30°C with shaking (200 rpm).

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A 30 litre fermenter with 15 litre production medium was inoculated with 900 ml (corresponding to 3 flasks) of the resulting overnight preculture, and cultured at 30°C for 25 hours under continuous stirring (350 rpm) and aeration (15 10 l/min). After culturing, the mycelia was harvested from the resulting culture broth by filtration on a Whatman GF/B filter by suction, and washed with 3 litres of deionized water. The mycelium yield was 186 g (wet weight).

15 A part (50 g) of the resulting mycelial mat was suspended in 700 ml of 50 mM borate buffer (pH 10.0), and disrupted by ultrasonication (Branson, Sonifer 250) at 5°C (3 x 5 minutes). After disruption, the mycelia was removed by centrifugation (29,000 g for 15 minutes), the cell-free 20 extract (700 ml) was brought to 40% saturation with ammonium sulfate and the resulting precipitate was removed by centrifugation (29,000 g for 20 minutes). The ammonium sulfate concentration was then increased to 70% saturation to precipitate the enzyme. The resulting precipitate was collected and solubilized in 100 ml of 50 mM borate buffer (pH 10.0). The crude extract was then dialysed for 24 hours 25 against 5 l of 50 mM borate buffer (pH 10.0). After dialysis the insoluble matters in the crude extract were removed by centrifugation (18,000 x g for 10 minutes). The resulting 30 supernatant contained 8.7 units of glycerol oxidase activity per ml.

2. Production, extraction and purification of glycerol oxidase using *Aspergillus japonicus* ATCC 1042 cultivated in a 35 production medium containing 5% glycerol

A spore suspension of *Aspergillus japonicus* ATCC 1042 was

prepared as described above. A preculture was prepared by inoculating 1 ml of the resulting spore suspension into a flask (500 ml) containing 200 ml production medium (5.0 % glycerol, 0.25% yeast extract, 0.1% Malt extract, 0.7% anti-foam (Contra spum), pH adjusted to 6.2 with HCl, sterilization at 121°C for 90 minutes). The preculture was incubated 3 days at 30°C with continuous shaking (200 rpm). A 6 litre fermenter with 5 litre production medium as described above was inoculated with 50 ml of the resulting 10 preculture and cultured at 30°C for 3 days under continuous stirring (250 rpm) and aeration (5 l/min). After culturing the mycelia was harvested from the resulting culture broth by filtration on a Whatman GF/B filter by suction, and washed with 3 litre ionized water containing 0.9% NaCl. 15 The resulting mycelia mat was frozen in liquid nitrogen, suspended in 200 ml of 50 mM phosphate buffer (pH 7.0) and disrupted by ultrasonication (Branson, Sonifer 250) at 5°C (4 minutes). After disruption, the mycelia was removed by filtration on a Whatman GF/A filter by suction. The enzyme in 20 the resulting filtrate was concentrated on a AMICON® 8400 ultrafiltration unit and contained 87 units of glycerol oxidase per ml after ultrafiltration.

3. Production, extraction and purification of glycerol
25 oxidase using *Aspergillus japonicus* ATCC 1042 cultivated in a
production medium containing 10% glycerol

A spore suspension of *Aspergillus japonicus* ATCC 1042 was prepared as described above. A 1 ml sample of the resulting 30 spore suspension was inoculated into each of 5 flasks (500 ml) with 200 ml production medium containing 10.0 % of glycerol, 0.1% of yeast extract and 0.1% of malt extract (pH adjusted to 6.2 with HCl, sterilization at 121°C for 15 minutes). The cultures were incubated for 5 days at 30°C with 35 shaking (140 rpm).

The extraction and concentration of the enzyme was carried

out as described above. The resulting filtrate contained 66 units of glycerol oxidase per ml after ultrafiltration.

4. Production of glycerol oxidase from *Penicillium funiculosum* and *Penicillium janthinellum*

Spore suspensions of *Penicillium funiculosum* NRRL 1132 and *Penicillium janthinellum* NRRL 2016 were prepared as described above. A 1 ml sample of each of the resulting spore suspensions was inoculated into separate flasks (1000 ml) containing 100 g wheat bran and 100 ml water (two flasks for each culture).

Glycerol oxidase was extracted by suspending the wheat bran cultures in 900 ml of 30 mM phosphate buffer (pH 6.5) containing 0.1% Triton X100 (Merck). The mycelial mat was removed from the cultivation media by filtration using a Whatman GF/B filter. The resulting mycelia mat was frozen in liquid nitrogen, suspended in 200 ml of 50 mM phosphate buffer (pH 7.0) and disrupted by ultrasonication (Branson, Sonifer 250) at 5°C (4 minutes). After disruption, the mycelia was removed by filtration on a Whatman GF/A filter by suction. The resulting filtrate from the *Penicillium funiculosum* culture contained 7.4 units of glycerol oxidase per ml, and the resulting filtrate from the *Penicillium janthinellum* culture contained 11.3 units of glycerol oxidase per ml.

B. PRODUCTION, PURIFICATION AND CHARACTERIZATION OF *ASPERGILLUS TUBIGENSIS* LIPASE 3

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Materials and Methods

(i) Determination of lipase activity and protein

35 1. Plate assay on tributyrin-containing medium

The assay is modified from Kouker and Jaeger (Appl. Environ. Microbiol., 1987, 53:211-213).

A typical protocol for this assay is as follows: 100 ml 2% agar in 50 mM sodium phosphate buffer (pH 6.3) is heated to boiling, and after cooling to about 70°C under stirring, 5 ml 10 0.2% Rhodamine B is added under stirring plus 40 ml of tributyrin. The stirring is continued for 2 minutes. The mixture 15 is then sonicated for 1 minute. After an additional 2 minutes of stirring, 20 ml of the agar mixture is poured into individual petri dishes. In the absence of lipase activity, the 20 agar plates containing tributyrin and Rhodamine B will appear opaque and are pink coloured.

To quantify lipase activity, holes having a diameter of 3 mm are punched in the above agar and filled with 10 μ l of lipase 15 preparation. The plates are incubated for varying times at 37°C. When lipase activity is present in the applied preparation to be tested, a sharp pink/reddish zone is formed around the holes. When the plates are irradiated with UV light at 350 nm, the lipase activity is observed as halos of orange 20 coloured fluorescence.

2. Modified Food Chemical Codex assay for lipase activity

Lipase activity based on hydrolysis of tributyrin is measured 25 according to Food Chemical Codex, Forth Edition, National Academy Press, 1996, p. 803. With the modification that the pH is 5.5 instead of 7. One LUT (lipase unit tributyrin) is defined as the amount of enzyme which can release 2 μ mol butyric acid per min. under the above assay conditions.

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3. *p*-nitrophenyl acetate assay

Lipase activity can also be determined colorimetrically using *p*-nitrophenyl acetate as a substrate e.g. using the following 35 protocol: In a microtiter plate 10 μ l of sample or blank is added followed by the addition of 250 μ l substrate (0.5 mg *p*-nitrophenyl acetate per ml 50 mM phosphate buffer, pH 6.0).

The microtiter plate is incubated for 5 minutes at 30°C and the absorbance at 405 nm is read using a microplate reader. 1 unit is defined as 1 μ mol *p*-nitrophenol released per 5 minutes.

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4. *p*-nitrophenyl hexanoate assay

Lipase activity can be determined by using *p*-nitrophenyl hexanoate as a substrate. This assay is carried out by adding 10 10 μ l of sample preparation or blank to a microtiter plate followed by the addition of 250 μ l substrate (0.5 mg *p*-nitrophenyl hexanoate per ml of 20 mM phosphate buffer, pH 6.). At this concentration of substrate the reaction mixture appears as a milky solution. The microtiter plate is incubated for 5 15 minutes at 30°C and the absorbance at 405 nm is read in a microplate reader.

5. Titrimetric assay of lipase activity

20 Alternatively, lipase activity is determined according to Food Chemical Codex (3rd Ed., 1981, pp 492-493) modified to sunflower oil and pH 5.5 instead of olive oil and pH 6.5. The lipase activity is measured as LUS (lipase units sunflower) where 1 LUS is defined as the quantity of enzyme 25 which can release 1 μ mol of fatty acids per minute from sunflower oil under the above assay conditions.

6. Protein measurement

30 During the course of purification of lipase as described in the following, the protein eluted from the columns was measured by determining absorbance at 280 nm. The protein in the pooled samples was determined in microtiter plates by a sensitive Bradford method according to Bio-Rad (Bio-Rad 35 Bulletin 1177 EG, 1984). Bovine serum albumin was used as a standard.

EXAMPLE 2

Production, purification and characterization of lipase 35 2.1. Production

A mutant strain of *Aspergillus tubigensis* was selected and used for the production of wild type lipase. This lipase is referred to herein as lipase 3. The strain was subjected to a 10 fermentation in a 750 l fermenter containing 410.0 kg of tap water, 10.8 kg soy flour, 11.1 kg ammonium monohydrogenphosphate, 4.0 kg phosphoric acid (75%), 2.7 kg magnesium sulfate, 10.8 kg sunflower oil and 1.7 kg antifoam 1510. The substrate was heat treated at 121°C for 45 minutes. The 15 culture media was inoculated directly with 7.5×10^9 spores of the mutant strain. The strain was cultivated for three days at 38°C, pH controlled at 6.5, aeration at 290 l/min and stirring at 180 rpm the first two days and at 360 rpm the last day. The fermentate was separated using a drum filter 20 and the culture filtrate was concentrated 3.8 times by ultra-filtration. The concentrated filtrate was preserved with potassium sorbate (0.1%) and sodium benzoate (0.2%) and used as a starting material for purification of lipase.

25 2.2. Purification of lipase

A 60 ml sample of ferment (cf. 2.1) containing 557 LUS/ml, pH 5.5 was first filtered through a GF/B filter and subsequently through a 0.45 µm filter. The filtered sample was desalted 30 using a Superdex G25 SP column (430 ml, 22 x 5 cm) equilibrated in 20 mM triethanolamine, pH 7.3. The flow rate was 5 ml/min. The total volume after desalting was 150 ml.

The desalted sample was applied to a Source Q30 anion ex- 35 changer column (100 ml, 5x5 cm) equilibrated in 20 mM triethanolamine, pH 7.3. The column was washed with equilibration buffer until a stable baseline was obtained.

Lipase activity was eluted with a 420 ml linear gradient from 0 to 0.35 M sodium chloride in equilibration buffer, flow rate 5 ml/min. Fractions of 10 ml were collected. Sodium acetate (100 μ l of a 2M solution) was added to each fraction 5 to adjust pH to 5.5. Fractions 26-32 (70 ml) were pooled.

To the pool from the anion exchange step was added ammonium sulfate to 1 M and the sample was applied to a Source Phenyl HIC column (20 ml, 10x2 cm) equilibrated in 20 mM sodium acetate (pH 5.5), 1 M ammonium sulfate. The column was washed with the equilibration buffer. Lipase was eluted with a 320 ml linear gradient from 1 M to 0 M ammonium sulfate in 20 mM sodium acetate (pH 5.5), flow 1.5 ml/min. Fractions of 7.5 ml were collected.

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Fractions 33-41 were analyzed by SDS-PAGE using a NOVEX system with precast gels. Both electrophoresis and silver staining of the gels were done according to the manufacturer (Novex, San Diego, USA). (The same system was used for native 20 electrophoresis and isoelectric focusing). It was found that fraction 40 and 41 contained lipase as the only protein.

2.3. Characterization of the purified lipase

25 (i) Determination of molecular weight

The apparent molecular weight of the native lipase was 37.7 kDa as measured by the above SDS-PAGE procedure. The purified lipase eluted at a molecular weight of 32.2 kDa from a Super-30 rose 12 gel filtration column (50 mM sodium phosphate, 0.2 M sodium chloride, pH 6.85, flow 0.65 ml/min) and is therefore a monomer.

The molecular weight of the lipase was also determined by 35 matrix-assisted laser desorption ionisation (MALDI) by means of a time-of-flight (TOF) mass spectrometer (Voyager Bio-Spectrometry Workstation, Perspective Biosystems). Samples were prepared by mixing 0.7 μ l of desalted lipase solution

and 0.7 μ l of a matrix solution containing sinapic acid (3.5-dimethoxy-4-hydroxy cinnamic acid) in 70% acetonitrile (0.1% TFA, 10 mg/ml). 0.7 μ l of the sample mixture was placed on top of a stainless steel probe tip and allowed to air-dry 5 prior to introduction into the mass spectrometer. Spectra were obtained from at least 100 laser shots and averaged to obtain a good signal to noise ratio. The molecular mass for the lipase was found to be 30,384 Da and 30,310 Da by two independent analyses.

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Digestion of the lipase with endo- β -N-acetyl-glucosaminidase H (10 μ l) from *Streptomyces* (Sigma) was carried out by adding 200 μ l lipase and incubating at 37°C for 2 hours. The digestion mixture was desalted using a VSWP filter and analyzed 15 directly by MALDI mass spectrometry. A major component of deglycosylated lipase gave a mass of 29,339 Da and 29,333 Da by two independent analyses. A minor component with a mass of 29,508 Da was also observed. These values corresponds well to the later calculated theoretical value of 28,939 Da based on 20 the complete amino acid sequence of the mature lipase.

(ii) Determination of the isoelectric point

The isoelectric point (pI) for the lipase was determined by 25 isoelectric focusing and was found to be 4.1.

A calculation of the pI based on the amino acid sequence as determined in the following and shown as SEQ ID NO: 9 gave an estimated pI of 4.07.

30

(iii) Determination of temperature stability

Eppendorf tubes with 25 μ l of purified lipase 3 plus 50 μ l 100 mM sodium acetate buffer (pH 5.0) were incubated for 1 35 hour in a water bath at respectively 30, 40, 50, and 60°C. A control was treated in the same way, but left at room temperature. After 1 hour the lipase 3 activity was determined by the p-nitrophenyl acetate assay as described above.

The purified lipase had a good thermostability. It was found that the lipase maintained 60% of its activity after 1 hour at 60°C. 80% and 85% activity was maintained after 1 hour at 50°C and 40°C respectively.

(iv) Determination of pH stability

Purified lipase 3 (200 µl) was added to 5 ml of 50 mM buffer 10 solutions: (sodium phosphate, pH 8.0, 7.0 and 6.0 and sodium acetate pH 5.0, 4.0 and 3.5). The control was diluted in 5 ml of 4 mM sodium acetate pH 5.5. After four days at room temperature the residual activity was measured by the Modified Food Chemical Codex assay for lipase activity as described 15 above. The lipase was very stable in the pH range from 4.0 to 7.0 where it maintained about 100% activity relative to the control (Table 2.1). At pH 3.5 the lipase maintained 92% activity, and at pH 8.0 95% residual activity was maintained as compared to the control.

20

Table 2.1. pH stability of lipase 3

pH	Activity (LUT/ml)	Activity (%)
Control (pH 5.5)	89.2	100
3.5	82.5	92
4.0	91.7	103
5.0	86.5	97
6.0	92.4	104
7.0	90.6	102
8.0	84.4	95

EXAMPLE 3

Amino acid sequencing of lipase 3

5 Purified lipase enzyme was freeze-dried and 100 µg of the freeze-dried material was dissolved in 50 µl of a mixture of 8 M urea and 0.4 M ammonium hydrogencarbonate, pH 8.4. The dissolved protein was denatured and reduced for 15 minutes at 50°C following overlay with nitrogen and addition of 5 µl 45 10 mM dithiothreitol. After cooling to room temperature, 5 µl of 100 mM iodoacetamide was added for the cysteine residues to be derivatized for 15 minutes at room temperature in the dark under nitrogen.

15 135 µl of water and 5 µg of endoproteinase Lys-C in 5 µl of water was added to the above reaction mixture and the digestion was carried out at 37°C under nitrogen for 24 hours. The resulting peptides were separated by reverse phase HPLC 20 on a VYDAC C18 column (0.46 x 15 cm; 10 µm; The Separation Group, California, USA) using solvent A: 0.1% TFA in water and solvent B: 0.1% TFA in acetonitrile. Selected peptides were rechromatographed on a Develosil C18 column (0.46 x 10 cm, Novo Nordisk, Bagsværd, Denmark) using the same solvent system, prior to N-terminal sequencing. Sequencing was done 25 using an Applied Biosystems 476A sequencer using pulsed-liquid fast cycles according to the manufacturer's instructions (Applied Biosystems, California, USA).

For direct N-terminal sequencing, the purified protein was 30 passed through a Brownlee C2 Aquapore column (0.46 x 3 cm, 7 µm, Applied Biosystems, California, USA) using the same solvent system as above. N-terminal sequencing was then performed as described above. As the protein was not derivatized prior to sequencing, cysteine residues could not be 35 determined.

The following peptide sequences were found:

N-terminal: Ser-Val-Ser-Thr-Ser-Thr-Leu-Asp-Glu-
Leu-Gln-Leu-Phe-Ala-Gln-Trp-Ser-Ala-
Ala-Ala-Tyr-X-Ser-Asn-Asn
(SEQ ID NO:1)

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Internal peptide 1: Val-His-Thr-Gly-Phe-Trp-Lys
(SEQ ID NO:2)

Internal peptide 2: Ala-Trp-Glu-Ser-Ala-Ala-Asp-Glu-Leu-
10 Thr-Ser-Lys-Ile-Lys
(SEQ ID NO:3)

No further peptides could be purified from the HPLC fractionation presumably because they were very hydrophobic 15 and therefore tightly bound to the reverse phase column.

A search in SWISS-PROT database release 31 for amino acid sequences with homology to the above peptides was performed and only three sequences were found.

20 All of the above peptides showed a low homology to the above known sequences. Especially internal peptide 2 has very low homology to the three lipases, LIP-RHIDL, LIP-RHIMI and MDLA-PENCA from *Rhizopus delamar* (Haas and Berka, Gene, 1991, 25 109:107-113), *Rhizomucor miehei* (Boel et al., Lipids, 1988, 23:701-706) and *Penicillium camembertii* (Yamaguchi et al., Gene, 1991, 103:61-67; Isobe and Nokihara, Febs. Lett., 1993, 320:101-106) respectively. Although the homology was not very high it was possible to position the lipase 3 peptides on 30 these sequences as it is shown in the below Table 3.1.

Table 3.1. Alignment of lipase 3 peptides with known lipase sequences

EXAMPLE 4

Isolation and purification of *Aspergillus tubigensis* genomic DNA

5 The *Aspergillus tubigensis* mutant strain was grown in PDB (Difco) for 72 hours and the mycelium was harvested. 0.5-1 g of mycelium was frozen in liquid nitrogen and ground in a mortar. Following evaporation of the nitrogen, the ground 10 mycelium was mixed with 15 ml of an extraction buffer (100 mM Tris·HCl, pH 8.0, 50 mM EDTA, 500 mM NaCl, 10 mM β -mercaptoethanol) and 1 ml 20 % sodium dodecylsulfate. The mixture was vigorously mixed and incubated at 65°C for 10 min. 5 ml 3M potassium acetate, (pH 5.1 adjusted with glacial acetic acid) 15 was added and the mixture further incubated on ice for 20 min. The cellular debris was removed by centrifugation for 20 min. at 20,000 \times g and 10 ml isopropanol was added to the supernatant to precipitate (30 min at -20°C) the extracted DNA. After further centrifugation for 15 min at 20,000 \times g, 20 the DNA pellet was dissolved in 1 ml TE (10 mM Tris·HCl pH 8.0, 1 mM EDTA) and precipitated again by addition of 0.1 ml 3 M NaAc, pH 4.8 and 2.5 ml ethanol. After centrifugation for 15 min at 20,000 \times g the DNA pellet was washed with 1 ml 70 % ethanol and dried under vacuum. Finally, the DNA was dissolved 25 in 200 μ l TE and stored at -20°C.

EXAMPLE 5

The generation of a fragment of the putative gene coding for 30 lipase 3 using PCR

To obtain a fragment of the putative gene (in the following referred to as the *lipA* gene) as a tag to isolate the complete gene, a PCR amplification procedure based on the information in the isolated peptide sequences was carried out. 35

Degenerated primers for PCR amplification of a fragment of

the lipase gene were designed based on the amino acid sequences of the isolated peptides. The following three PCR primers were synthesised:

5 C035: TTC CAR AAN CCN GTR TGN AC (SEQ ID NO:4)

20 mer 256 mixture, based on peptide 1 sequence VHTGFWK (Reversed).

10 C036: CAR YTN TTY GCN CAR TGG (SEQ ID NO:5)

18 mer 256 mixture, based on the N-terminal sequence QLFAQW.

C037: GCV GCH SWY TCC CAV GC (SEQ ID NO:6)

15 17 mer 216 mixture, based on internal peptide 2 sequence AWESAA (reversed).

The oligonucleotides were synthesised on a Applied Biosystems 20 model 392 DNA/RNA Synthesizer. To reduce the degree of degeneracy the rare Ala codon GCA and the Ser codon TCA have been excluded in design of primer C037.

With these primers the desired fragments were amplified by 25 PCR. Using these primers it was expected that a fragment of about 300 bp should be amplified provided there are no introns in the fragment.

The following PCR reactions were set up in 0.5 ml PCR tubes 30 to amplify a putative *lipA* fragment:

1. 0.5 μ g total genomic DNA,
100 pmol primer C036,
100 pmol primer C037,
35 10 μ l PCR Buffer II (Perkin Elmer),
6 μ l 25 mM MgCl₂,
2 μ l dNTP mix (10 mM dATP, 10 mM dCTP, 10 mM dGTP, 10 mM dTTT
2 units AmpliTaq polymerase (Perkin Elmer), and

water to a total volume of 100 μ l.

2. 0.5 μ g total genomic DNA,
100 pmol primer C035,
5 100 pmol primer C036,
10 μ l PCR Buffer II (Perkin Elmer),
6 μ l 25 mM MgCl₂,
2 μ l dNTP mix (10 mM dATP, 10 mM dCTP, 10 mM dGTP, 10 mM dTT
2 units AmpliTaq polymerase (Perkin Elmer), and
10 water to a total volume of 100 μ l.

The reactions were performed using the following program:

94°C 2 min
94°C 1 min)
15 40°C 1 min)
72°C 1 min) These three steps were repeated for 30
72°C 5 min cycles
5°C SOAK

20 The PCR amplifications were performed in a MJ Research Inc.
PTC-100 Thermocycler.

In reaction 1, three distinct bands of about 300, 360 and 400
bp, respectively could be detected. These bands were isolated
25 and cloned using the pT7-Blue-T-vector kit (Novagene). The
sizes of these fragment is in agreement with the expected
size provided that the fragment contains 0, 1 or 2 introns,
respectively.

30 The three fragments were sequenced using a "Thermo Sequenase
fluorescent labelled primer cycle sequencing Kit" (Amersham)
and analyzed on a ALF sequencer (Pharmacia) according to the
instructions of the manufacturer. The fragment of about 360
bp contained a sequence that was identified as a lipase and,
35 as it contained the part of the N-terminal distal to the
sequence used for primer design, it was concluded that the
desired *lipA* gene fragment was obtained.

The sequence of the about 360 bp PCR fragment (SEQ ID NO:7) is shown in the following Table 5.1. The peptide sequence used for primer design is underlined. The remaining part of the N-terminal sequence is doubly underlined.

Table 5.1. PCR-generated putative *lipA* sequence

	10	20	30	40	50	60	
10							
tacccggggntccgatt <u>CAGTTGTCGCGCAATGGTCTGCCGCAGCTTATTGCTCGAATA</u>							
	15	70	80	90	100	110	120
ATATCGACTCGAAAGAVTCCAATTGACATGCACGGCCAACGCCGTCCATCAGTCGAGG							
	20	N I D S K X S N L T C T A N A C P S V E					
		130 140 150 160 170 180					
AGGCCAGTACACAGATGCTGGAGTTGACCTGTATGTCACTCAGATCGCAGACATAG							
	25	E A S T T M L L E F D L Y V T Q I A D I					
		190 200 210 220 230 240					
AGCACAGCTAATTGAAACAGGACGAACGACTTTGGAGGCACAGCCGTTCTGGCCGCG							
	30	E H S - L N R T N D F W R H S R F P G R					
		250 260 270 280 290 300					
GACAACACCAACAAAGCGGCTCGTGGTCGCCCTCCGGGGAGCAGCACGATTGAGAACTGG							
	35	G Q H Q Q A A R G R L P G K Q H D - E L					
		310 320 330					
ATTGCTAATCYTGACTTCATCTGGRAGATAACG							
	40	D C - X - L H P X R -					

The finding of this sequence permitted full identification of the PCR fragment as part of the *lipA* gene. The stop codon found in the reading frame can be caused either by a PCR or a reading error or there can be an intron encoded in the fragment as a consensus intron start and ending signal (shown in bold). If the putative intron is removed a shift in reading frame will occur. However, an alignment of the deduced amino acid sequence and the fungal lipases shown in Table 3.1 suggested that the fragment was part of the desired gene.

10

EXAMPLE 6

Cloning and characterisation of the *lipA* gene

15 (i) Construction of an *Aspergillus tubigensis* genomic library

Aspergillus tubigensis genomic DNA was digested partially with Tsp5091 (New England Biolabs Inc.). 10 µg DNA was digested in 100 µl reaction mixture containing 2 units Tsp5091.

20 After 5, 10, 15 and 20 minutes 25 µl was removed from the reaction mixture and the digestion was stopped by addition of 1 µl 0.5 M EDTA, pH 8.0. After all four reactions had been stopped, the samples were run on a 1% agarose gel in TAE buffer (10 x TAE stock containing per litre: 48.4 g Trizma base, 11.5 ml glacial acetic acid, 20 ml 0.5 M EDTA pH 8.0).

25 *Hind*III-digested phage Lambda DNA was used as molecular weight marker (DNA molecular weight marker II, Boehringer, Mannheim). Fragments of a size between about 5 and 10 kb were cut out of the gel and the DNA fragments were purified using 30 Gene Clean II Kit (Bio-101 Inc.). The purified fragments were pooled and 100 ng of the pooled fragments were ligated into 1 µg EcoRI-digested and dephosphorylated ZAP II vector (Stratagene) in a total volume of 5 µl. 2 µl of this volume was packed with Gigapack II packing extract (Stratagene) which 35 gave a primary library of 650,000 pfu.

E. coli strain XL1-Blue-MRF (Stratagene) was infected with 5x 50,000 pfu of the primary library. The infected bacteria were

mixed with top agarose (as NZY plates but with 6 g agarose per litre instead of the agar) and plated on 5 NZY plates (13 cm). After incubation at 37°C for 7 hours, 10 ml SM buffer (per litre: 5.8 g NaCl, 2.0 g MgCl₂·7H₂O, 50 ml 1 M Tris·HCl 5 pH 7.5, 5.0 ml of 2% (w/v) gelatine) and incubated overnight at room temperature with gently shaking. The buffer containing washed-out phages was collected and pooled. 5% chloroform was added and after vigorous mixing the mixture was incubated 1 hour at room temperature. After centrifugation for 2 minutes at 10,000 x g the upper phase containing the amplified library was collected and dimethylsulphoxide was added to 7%. Aliquots of the library was taken out in small tubes and frozen at -80°C. The frozen library contained 2.7×10^9 pfu/ml with about 6% without inserts.

15

(ii) Screening of the *Aspergillus tubigensis* library

2 x 50.000 pfu were plated on large (22 x 22 cm) NZY plates containing a medium containing per litre: 5 g NaCl, 2 g 20 MgSO₄·7H₂O, 5 g yeast extract, 10 g casein hydrolysate, 15 g agar, pH adjusted to 7.5 with NaOH. The medium was autoclaved and cooled to about 60°C and poured into the plates. Per plate was used 240 ml of medium.

25 The inoculated NZY plates were incubated overnight at 37°C and plaque lifts of the plates were made. Two lifts were made for each plate on Hybond N (Amersham) filters. The DNA was fixed using UV radiation for 3 min. and the filters were hybridized as described in the following using, as the probe, 30 the above PCR fragment of about 360 bp that was labelled with ³²P-dCTP using Ready-to-Go labelling kit (Pharmacia).

The filters were prehybridised for one hour at 65°C in 25 ml prehybridisation buffer containing 6.25 ml 20 x SSC (0.3 M 35 Na₃citrate, 3 M NaCl), 1,25 ml 100 x Denhard solution, 1.25 ml 10% SDS and 16.25 ml water. 150 µl 10 mg/ml denatured Salmon sperm DNA was added to the prehybridization buffer

immediately before use. Following prehybridization, the prehybridisation buffer was discarded and the filters hybridised overnight at 65°C in 25 ml prehybridisation buffer with the radiolabelled PCR fragment.

5

Next day the filters were washed according to the following procedure: 2 x 15 min. with 2 x SSC + 0.1 % SDS, 15 min. with 1 x SSC + 0.1 % SDS and 10 min. with 0.1 x SSC + 0.1% SDS.

10 All washes were done at 65°C. The sheets were autoradiographed for 16 hours and positive clones were isolated. A clone was reckoned as positive only if there was a hybridisation signal on both plaque lifts of the plate in question.

15 Seven putative clones were isolated and four were purified by plating on small petri dishes and performing plaque lifts essentially as described above.

The purified clones were converted to plasmids using an
20 ExAssist Kit (Stratagene).

Two sequencing primers were designed based on the about 360 bp PCR fragment. The sequencing primers were used to sequence the clones and a positive clone with the *lipA* gene encoding
25 lipase 3 was found. The isolated positive clone was designated pLIP4.

(iii) Characterisation of the pLIP4 clone

30 A restriction map of the clone was made. The above 360 bp PCR fragment contained a *SacII* site and as this site could be found in the genomic clone as well this site facilitated the construction of the map. The restriction map showing the structure of pLIP4 is shown in Fig. 1. The restriction map
35 shows that the complete gene is present in the clone. Additionally, since promoter and terminator sequences are present, it was assumed that all the important regions is pre-

sent in the clone.

A sample of *Escherichia coli* strain DH5 α containing pLIP4 was deposited in accordance with the Budapest Treaty with The 5 National Collections of Industrial and Marine Bacteria Limited (NCIMB) at 23 St. Machar Drive, Aberdeen, Scotland, United Kingdom, AB2 1RY on 24 February 1997 under the accession number NCIMB 40863.

10 The gene was sequenced using cycle sequencing and conventional sequencing technology. The complete sequence (SEQ ID NO:8) is shown below in Table 6.1. The sequence has been determined for both strands for the complete coding region and about 100 bp upstream and downstream of the coding 15 region. The sequences downstream to the coding region have only been determined on one strand and contains a few uncertainties. In the sequence as shown below, the intron sequences are indicated as lowercase letters and the N-terminal and the two internal peptides (peptide 1 and peptide 2) 20 are underlined:

Table 6.1. The DNA sequence for the *lipA* gene and flanking sequences

25 1 CCNDTTAATCCCCACCGGGTTCCCGCTCCGGATGGAGATGGGGCCAAAATGGCAAC
 61 CCCCAGCTGGCAACCGAACACCCGGACCCGGACAAAGGATGGGATGAGGAGATA
 121 GGTGCCTGATTGATGGCTGGCTTCATCTGCTATCGTGCAGTGTCTTGGGTGAATAT
 181 TGTGTCGACTTACCCGGCTTCTGCTTTTCCCCCTGAGGCCCTGATGGGGAAATCGC
 241 GGTGGGTAATATGATATGGATAAAAGGGAGTCGGAGGTGCAAGTTGGATGAGGCAGT
 30 301 GTGTGTGTGTCATTGCAAGGCCGTTGGTCGCAAGGTTTGGAGTGCTTTGACAGCGCTGCTGGCTG
 361 TATACCGCAAGATGTTCTCTGGACGTTTGGAGTGCTTTGACAGCGCTGCTGGCTG
 M F S G R F G V L L T A L A A L
 421 GTGCTGCCGGCCGGCACCGCTTGCTGTGCGGAGtaggtgtccgatgtgagatggtt
 G A A A P A P L A V R
 35 481 gatagcaactgatgaagggtgaatagGTGTCTCGACTTCCACGTTGGATGAGTTGCAATTG
 S V S T S T L D E L Q L
 541 TTGCGCAATGGTCTGGCGCAGCTTATTGCTCGAATAATATCGACTCGAAAGACTCCAAC
 F A Q W S A A A Y C S N N I D S K D S N
 601 TTGACATGCACGGCCAACGCCCTGTCATCAGTCGAGGAGGCCAGTACACGATGCTGCTG
 40 L T C T A N A C P S V E E A S T T M L L
 661 GAGTTGACCTgtatgtcaactcagatcgacagacatagacagacatctaattgaaacagGAC

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      E F D L T
721  GAACGACTTTGGAGGCACGGCGTTCTGGCGGGACAACACCAACAAGCGGCTCGT
      N D F G G T A G F L A A D N T N K R L V
781  GGTCGCCCTCCGGGAAGCAGCACGATTGAGAACTGGATTGCTAATCTTGACTTCATCCT
      V A F R G S S T I E N W I A N L D F I L
841  GGAAGATAACGACGACCTCTGACCCGGCTGCAAGGTCCATACTGGTTCTGGAAGGCATG
      E D N D D L C T G C K V H T G F W K A W
901  GGAGTCGGCTCCGAGCAGACTGACGAGCAAGTCAAGTCTGGATGAGCACGTATTGGG
      E S A A D E L T S K I K S A M S T Y S G
10 961  CTATACCCCTATACTTCACCGGGCACAGTTGGGGCGCGATTGGCTACGCTGGAGCGAC
      Y T L Y F T G H S L G G A L A T L G A T
1021 AGTTCTGCAAATGACGGATATAGCGTTGAGCTGgtgagtcctcacaaagggtatggag
      V L R N D G Y S V E L
1081 cgacaatcggaaacagacagtcataatAGCACCTATGGATGTCCTCGAACATCGGAACTAT
      Y T Y G C P R I G N Y
1141 GCGCTGGCTGAGCATAATCACCAAGTCAGGGATCTGGGCCAACCTCCGTGTTACACACTTG
      A L A E H I T S Q G S G A N F R V T H L
1201 AACGACATCGTCCCCGGGTGCCACCCATGGACTTGGATTCAAGTCAGCCAAAGTCGGAA
20  N D I V P R V P P M D F G F S Q P S P E
1261 TACTGGATCACCAGTGGCAATGGAGCCAGTGTCAAGCGCTCGGATATCGAACATCGAG
      Y W I T S G N G A S V T A S D I E V I E
1321 GGAATCAATCAACGGCGGAAATCAGGGAGAACCGTGAAGCAACGGTGAAGCTGGCTCACTTG
      G I N S T A G N A G E A T V S V V A H L
25 1381 TGGTACTTTTGGATTCGGAGTCGCTGCTATAACTAGACCGACTGTCAGATTAGTG
      W Y F F A I S E C L L -
1441 ACGGGAGAAGTGTACATAAGTAATTAGTATATAATCAGAGCAACCCAGTGGTGGTGTGG
1501 TGGTGAAGAAGAACACATTGAGTCCCATTACGKAGCAGWTAAGCACKTKGGAGGC
1561 GCTGGTCTCCACTTGGCAGTTGGCGGCCATCAATCATTTCTCTCTTACTTTCTG
30 1621 CCACACAACTCCCATCTGCCAGCTGTCGATCCCCGGTTGCAACAACTATGCCCTCC
1681 GGGGCCCTCCGTGGTCTCCTATATTATTCCATCCGACGGCCGACGTTTACCCCTAACCT
1741 GCGCCGCCGCAAAATCTCCCGAGTCGGTCAACTCCCTCGAACCGCCGCCGATCGACC
1801 TCACGACCCGACCGTCTGYGATYGTCCAACCG

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35 (iv) Analysis of the sequence of the complete gene

The peptide sequences obtained could all be found in the deduced amino acid sequence (see Table 5.1) which confirms again that the sequence found is the sequence of the lipase 3 gene. The gene was designated *lipA*.

The amino acid sequence was aligned with the three fungal lipases used to align the peptide sequences. The alignment is shown in Table 6.2.

Table 6.2 Alignment of the lipase 3 sequence with known fungal lipases

5	LIPASE3	MFSG-----RFGVLL-----	TALAA	15
	MDLA_PENCA	MRLS-----FFTAL-----	SAVAS	14
	LIP_RHIDL	MVSFISISQGVSLCLVSSMMLGSSAVPVGKSGSSNTAVSASDNAALPP		50
	LIP_RHIMI	MVLKQRANYLGFLLVFTALV--EAVPIKRQNSTVDS-----LPP		40
		*	...	
10	LIPASE3	L-----		16
	MDLA_PENCA	L-----		15
	LIP_RHIDL	LISSRCAPPSENKGSKSDLQAEPYNMQRNTEWYESHGGNLTSIGKRDNLV		100
	LIP_RHIMI	LIPSRSTSAPSSSPSTTDPEAPAM-----SRNGPLPS-----DVETK		76
15		*	...	
	LIPASE3	-----GAAAPAPLA-----VRSVSTSTLDELQLFAQWSAAA		47
	MDLA_PENCA	-----GYALPGKLQ-----SRDVSTSELQDFEFWVQYAAAS		46
	LIP_RHIDL	GGMTLDLPSDAPPISLSSSTSNSASDGKKVVAATTAQIQEFTKYAGIAATA		150
20	LIP_RHIMI	YGMALNATSYPSDSV----VQAMSIDGGIRAATSQEINELTYYTLSANS		121
	
	LIPASE3	YCSNNIDSK-DSNLCTCTANACPSVEEASTTMLEFDLTDFGGTAGFLAA		96
	MDLA_PENCA	YYEADYTAQVGDKLSCSKGNCPPEVATGATVSYDFS-DSTITDTAGYIAV		95
25	LIP_RHIDL	YCRSVPV---GNWKDCVQ---CQKWPDPGKII---TFTSLLSDTNGYVLR		192
	LIP_RHIMI	YCRTVIP---GATWDCIH---CDA-TEDLKIH---TWSTLIYDTNAMVAR		162
		*
	LIPASE3	DNTNKRLLVAFRGSSSTIENWIANLDFILEDNDLCTGCKVHTGFWKAWES		146
30	MDLA_PENCA	DHTNSAVVLAFLRGSSYVRNNWADATEV--HTNPGLCDGCLAEIWFNSWWKL		144
	LIP_RHIDL	SDKQKTIYLVFRGRTNSFRSAITDVFNFNSDYKPV-KGAKVHAGFLSSYEQ		241
	LIP_RHIMI	GDSEKTIYIVFRGSSSIRNWIADLTFPVPSYPPV-SGTVKHGFLDSSYGE		211
	 * * * * * *		
35	LIPASE3	AAADELTSKIKSAMSTYSGTYLFTGHSLLGGALATLGATVL--RNDGY-SV		193
	MDLA_PENCA	VRDDIIKELKEVVAQNPVYELVVVGHSGAAVATLAATDL--RGKGYPSSA		192
	LIP_RHIDL	VVNDDYFPVVEQLTAHPTYKVIVTGHSGGGQALLAGMDLYQREFRLSPK		291
	LIP_RHIMI	VQNELVATVLDQFKQYPSYKVAVTGHSGGGATALLCALDLYQREEGLSSS		261
	 * * * * *		
40	LIPASE3	ELYTY--GCPRIGNYALAEHITSQGSGANFRVTHLNDIVPRVPPMDFGFS		241
	MDLA_PENCA	KLYAY--ASPRVGNALAKYITAQGN---NFRFTHTNDPVPKLPLLSMGYV		238
	LIP_RHIDL	NLSIFTVGGPRVGNPTFAYYVESTGIPFQ-RTVHKRDIVPHVPPQSFGFL		340
	LIP_RHIMI	NLFLYTQGQPRVGDPAFANYVVSSTGIPYR-RTVNERDIVPHLPPAAFGL		310
45		.* . . . * * * * *		

LIPASE3	QPSPEYWITSGNGASVTASDIEVIEGINSTAGNAGEATVSVV---AHLWY	288
MDLA_PENCA	HVSPEYWITSPNNATVTSSTDVKIDGDSFDGNTGTLPLLTDFEAHIWY	288
LIP_RHIDL	HPGVESWIKSGCTSN-VQICITSEIE-----TKDCNSNIVPFTSILDHLSY	383
5 LIP_RHIMI	HAGEEYWITDNPETVQVCTSDEL-----TSDCSNSNIVPFTSVDLHLSY	354
*.....*.....*.....*.....*.....*.....*.....*.....*	
LIPASE3	FFAISECL-----L	297
MDLA_PENCA	FVQVDAGKGPGPLPKRV	305
10 LIP_RHIDL	F-DINEGSC-----L	392
LIP_RHIMI	F-GINTGLC-----T	363
	*	

The above alignment shows that lipase 3 is homologous to the 15 known lipase sequences but that the homology is not very high. Deletions or insertions in the lipase 3 sequence was not observed when comparing the sequence with these three lipases. This strengthens the probability that the putative introns have been identified correctly.

20 A search in SWISS-PROT release 31 database was performed and
it did not lead to further sequences with higher homology
than that to the above known lipases (Table 6.3).

25 The sequence with highest homology is a mono- diacyl lipase from *Penicillium camembertii* where the identity is found to 42 %. However the C-terminal of lipase 3 resembles the 2 lipases from Zygomycetes (*Rhizopus* and *Rhizomucor*) and not the *P. camembertii* enzyme.

Table 6.3. Alignment of coding sequences of the *lipA* gene and gene coding for mono-diacyl lipase from *Penicillium camemberti*

35 LIPASE3 - MFSGRFVLLTALAALGAAAPAPLAVRSVSTSTLDELQLQFAQWSAAACYS - 50
 MELA, PENC1 - MFLISSETPL - SAVASVCGALPKLQSPRDVSTSELDFEETVQYRASVYK - 49

25 The N-terminal of the mature lipase has been determined by N-terminal sequencing to be the serine residue No. 28 of the lipase 3 precursor (SEQ ID NO:9) as shown in Table 6.4 below. Hence the amino acids No. 1 to No. 27 is the signal sequence.

30 Table 6.4: Amino acid sequence of the precursor of lipase 3

	5	10	15	20	25	30
35	1 M F S G R F G V L L T A L A A L G A A A P A P L A V R S V S					
	31 T S T L D E L Q L F A Q W S A A A Y C S N N I D S K D S N L					
	61 T C T A N A C P S V E E A S T T M L L E F D L T N D F G G T					
	91 A G F L A A D N T N K R L L V V A F R G S S T I E N W I A N L					
	121 D F I L E D N D D L C T G C K V H T G F W K A W E S A A D E					
	151 L T S K I K S A M S T Y S G Y T L Y F T G H S L G G A L A T					
40	181 L G A T V L R N D G Y S V E L Y F T Y G C P R I G N Y A L A E					
	211 H I T S Q G S G A N F R V T H L N D I V P R V P P M D F G F					
	241 S Q Q P S P E Y W I T S G N G A S V T A S D I E V I E G I N S					
	271 T A G N A G E F A T V S V V A H L W Y F T Y G C P R I G N Y A L A E					

Number of residues : 297

Residues 167-176 are recognised as a common motif for the serine lipases (PROSITE). The crystal structure for the *Rhizomucor miehei* serine lipase has been examined and the 5 residues in the active site identified (Brady et al., *Nature*, 1990, **343**:767-770; Derewanda et al., *J. Mol. Biol.*, 1992, **227**:818-839). The active site residues of *R. miehei* lipase have all been conserved in all the lipases and correspond to the following residues in lipase 3: serine 173, aspartic acid 10 228 and histidine 285.

Lipase 3 contains 7 cysteine residues. Four of these are conserved in the *P. camembertii* lipase where they form disulphide bonds (Isobe and Nokuhara, *Gene*, 1991, **103**:61-67). 15 This corresponds to disulphide bonds between residue 62- 67 and 131-134. In addition, two cysteine residues are homologous to two C residues which forms an additional disulphide bond in *Rhizopus* and *Rhizomucor* lipases corresponding to residues 49-295.

20 Two putative N-glycosylation sites were found in lipase 3 in position 59 and 269. Neither of these are conserved in the other fungal lipases.

25 EXAMPLE 7

Transformation of *Aspergillus tubigensis* and overexpression of lipase 3 in *A. tubigensis*

30 The protocol for transformation was based on the teachings of Buxton et al. (*Gene*, 1985, **37**:207-214), Daboussi et al (*Curr. Genet.*, 1989, **15**:453-456) and Punt and van den Hondel, (*Meth. Enzym.*, 1992, **216**:447-457).

35 A multicopy *lipA* strain was produced by transforming the pLIP4 plasmid into *Aspergillus tubigensis* strain 6M 179 using cotransformation with a hygromycin resistant marker plasmid.

A screening procedure used to visualise fungal lipase after ultrathin layer isoelectric focusing was adapted to screen *Aspergillus tubigensis* transformants grown on agar plates. Screening of lipase producers on agar plates was done using 5 2% olive oil as the substrate for the enzyme (lipase) as well as the inducer for the lipase promoter. In addition, the plates contained a fluorescent dye, Rhodamine B. In the presence of olive oil, the transformants will be induced to secrete lipase. The lipase secreted into the agar plate will 10 hydrolyse the olive oil causing the formation of orange fluorescent colonies that is visible upon UV radiation (350 nm). The appearance of fluorescent colonies was generally monitored after 24 hours of growth. After several days of growth, the lipase producing strains could be identified as 15 orange fluorescent strains that are visible by eye. Under this plate screening condition, the untransformed strain gave no background fluorescence and appeared as opaque pink colonies.

20 Sixteen transformants that showed orange fluorescent halos were cultivated for 8 days in shake flasks containing 100 ml of minimal medium supplemented with 1% olive oil, 0.5% yeast extract and 0.2% casamino acids. The amount of lipase secreted was quantified by applying 10 μ l of cell-free culture 25 supernatant into holes punched in olive oil- Rhodamine B agar plates and incubating the plates overnight at 37°C. Five transformants with higher lipase production were found.

The cell-free culture supernatants from the five transformants were desalted using NAP 5 columns (Pharmacia) and 30 equilibrated in 1M ammonium sulfate (50 mM sodium acetate, pH 5.5). The desalted culture supernatants were fractionated by hydrophobic interaction chromatography (HIC) on a Biogel Phenyl-5 PW column (Biorad). Elution was done by a descending 35 salt gradient of 1M to 0 M ammonium sulfate (20 mM sodium acetate, pH 5.5). A single discrete protein peak was observed after fractionation. The area of the protein peaks were calculated among the different transformants and compared

with the untransformed strain. The best transformant showed a 62-fold increase in the amount of lipase after HIC fractionation. A chromatogram of the HIC fractionated culture supernatant of this transformant is shown in Fig. 3 and a 5 similar chromatogram for the untransformed strain is shown in Fig. 4.

The fraction containing the transformed lipase was freeze-dried. The transformed lipase was carboxymethylated and sub-10 jected to N-terminal amino acid sequencing of the first 15 amino acids and it was found that the sequence of the recom-15 binant lipase was exactly the same as the native lipase indicating correct signal sequence cleavage.

15 The different lipase fractions collected after HIC were separated on a 12% Tris-Glycine SDS gel and silver staining revealed one protein band, confirming the homogeneity of the fractions. In addition, the crude extract showed a major 20 lipase band as the only band that accumulated in the culture supernatant in very high amounts when the fungus was cultured in the olive oil-containing medium.

The recombinant lipase was analysed by matrix-assisted laser desorption ionisation (MALDI) by means of a time-of-flight 25 (TOF) mass spectrometer as described hereinbefore. The molecular weight of the recombinant lipase was 32,237 Da.

Detection of N-linked oligosaccharides was achieved by digestion of the lipase with endo- β -N-acetyl-glucosaminidase H from 30 *Streptomyces* (Sigma). Digestion of recombinant lipase secreted into the growth medium altered the mobility of the band seen on SDS-PAGE which moved as a single band with a molecular mass of about 30 kDa.

35 Deglycosylated recombinant lipase generated by digestion with endoglycosidase and analysed directly by MALDI mass spectrometry gave a molecular weight of the polypeptide backbone of 29,325 Da.

C. BAKING EXPERIMENTS

EXAMPLE 8

5 Baking experiments using lipase 38.1. Baking procedures and analytical methods(i) Baking procedure for Danish toast bread

10

Flour (Danish reform flour) 2000 g, dry yeast 30 g, salt 30 g and water corresponding to 400 Brabender units + 3%, was kneaded in a Hobart Mixer with hook for 2 min. at low speed and 10 min. at high speed. Dough temperature after kneading

15

was 25°C. Resting time was 10 min. at 30°C. The dough was scaled 750 g per dough and rested again for 5 min at 33°C and 85% RH. After moulding on a Glimik moulder, the dough were proofed in tins for 50 min at 33°C and baked in a Wachtel oven for 40 min at 220°C with steam injection for 16 sec.

20

After cooling, the bread was scaled and the volume of the bread was measured by the rape seed displacement method. The specific volume is calculated by dividing the bread volume (ml) by the weight (g) of the bread.

25

The crumb was evaluated subjectively using a scale from 1 to 5 where 1 = coarsely inhomogeneous and 5 = nicely homogeneous.

30

Three breads baked in tins with lid were stored at 20°C and used for firmness measurements and pore measurements by means of an Image Analyzer.

(ii) Baking procedure for Danish rolls

35

Flour (Danish reform) 1500 g, compressed yeast 90 g, sugar 24 g, salt 24 g and water corresponding to 400 Brabender units - 2% were kneaded in a Hobart mixer with hook for 2 min. at low

speed and 9 min at high speed. After kneading, the dough temperature was 26°C. The dough was scaled 1350 g. After resting for 10 min. at 30°C, the dough was moulded on a Fortuna moulder after which the dough was proofed for 45 min. 5 at 34°C and baked in a Bago oven for 18 min. at 220°C with steam injection for 12 sec. After cooling, the rolls were scaled and the volume of the rolls was measured by the rape seed displacement method. Specific volume is calculated as described above.

10

(iii) Determination of pore homogeneity

The pore homogeneity of the bread was measured by means of an image analyzer composed of a standard CCD-video camera, a 15 video digitiser and a personal computer with WinGrain software. For every bread, the results of pore diameter in mm and pore homogeneity were calculated as an average of measurements from 10 slices of bread. The pore homogeneity was expressed in % of pores that are larger than 0.5 times the 20 average of pore diameter and smaller than 2 times the average diameter.

(iv) Determination of firmness

25 The firmness of bread, expressed as N/dm², was measured by means of an Instron UTM model 4301 connected to a personal computer. The conditions for measurement of bread firmness were:

Load Cell	Max. 100 N
30 Piston diameter	50 mm
Cross head speed	200 mm/min
Compression	25%
Thickness of bread slice	11 mm

35 The result was an average of measurements on 10 bread slices for every bread.

(v) Determination of gluten index

Gluten index was measured by means of a Glutomatic 2200 from Perten Instruments (Sweden). Immediately after proofing, 15 g of dough was scaled and placed in the Glutomatic and washed with 500 ml 2% NaCl solution for 10 min. The washed dough was transferred to a Gluten Index Centrifuge 2015 and the two gluten fractions were scaled and the gluten index calculated according to the following equation:

10

Gluten index = (weight of gluten remaining on the sieve x 100)/total weight of gluten

(vi) Extraction of lipids from dough

15

30 g of fully proofed dough was immediately frozen and freeze-dried. The freeze-dried dough was milled in a coffee mill and passed through a 235 μm screen. 4 g freeze-dried dough was scaled in a 50 ml centrifuge tube with screw lid 20 and 20 ml water saturated n-butanol (WSB) was added. The centrifuge tube was placed in a water bath at a temperature of 100°C for 10 min. after which the tubes were placed in a Rotamix and turned at 45 rpm for 20 min. at ambient temperature. The tubes were again placed in the water bath for 10 25 min. and turned on the Rotamix for another 30 min. at ambient temperature.

The tubes were centrifuged at 10,000 x g for 5 min. 10 ml of the supernatant was pipetted into a vial and evaporated to dryness under nitrogen cover. This sample was used for HPLC analysis.

A similar sample was fractionated on a Bond Elut Si (Varian 1211-3036). The non-polar fraction was eluted with 10 ml cyclohexan:isopropanol:acetic acid (55:45:1) and evaporated to dryness. This sample was used for GLC analysis.

(vii) HPLC analysis

Column: LiChrospher 100 DIOL 5 μ m (Merck art. 16152) 250x4 mm with a water jacket of a temperature of 50°C.

5

Mobile phases:

A: heptan:isopropanol:n-butanol:tetrahydrofuran:isooctan:water (64.5:17.5:7:5:5:1)

10 B: isopropanol:n-butanol:tetrahydrofuran:isooctan:water (73:7:5:5:10)

The mobile phases contained 1 mmol trifluoroacetic acid per 1 mobile phase and were adjusted to pH 6.6 with ammonia.

15

Pump: Waters 510 equipped with a gradient controller.

Gradient:

	Flow (ml/min)	Time (min)	A (%)	B (%)
20	1.0	0	100	0
	1.0	25	0	100
	1.0	30	0	100
	1.0	35	100	0
	1.0	40	100	0

25

Detector: CUNOW DDL21 (evaporative light-scattering); temperature 100°C; voltage: 600 volt; air flow: 6.0 l/min.

Injector: Hewlett Packard 1050; injection volume: 50 μ l.

30

The samples for analysis were dissolved in 5 ml chloroform:methanol (75:25), sonicated for 10 min and filtered through a 0.45 μ m filter.

35 (viii) GLC analysis

Perkin Elmer 8420 Capillary Gas Chromatograph equipped with WCOT fused silica column 12.5 m x 0.25 mm coated with 0.1 μ m

stationary phase of 5% phenyl-methyl-silicone (CP Sil 8 CB from Crompack).

Carrier: Helium

Injection: 1.5 μ l with split

5 Detector: FID 385°C

Oven program:	1	2	3	4
Oven temperature, °C	80	200	240	360
Isothermal time, min	2	0	0	10
Temperature rate, °C/min	20	10	12	--

10

Sample preparation: 50 mg non-polar fraction of wheat lipids was dissolved in 12 ml heptane:pyridine (2:1) containing 2 mg/ml heptadecane as internal standard. 500 μ l of the solution was transferred to a crimp vial and 100 μ l N-methyl-N-trimethylsilyl-trifluoracetamide was added. The mixture was allowed to react for 15 min at 90°C.

Calculation: Response factors for mono-, di- and triglycerides and free fatty acids were determined from 20 reference mixtures of these components. Based on these response factors, the glycerides and the free fatty acids were calculated in wheat lipids.

8.2. Baking experiments with lipase 3 in Danish toast bread

25

The effect of adding lipase 3 to a dough for making Danish toast bread was evaluated. The enzyme was added as a freeze-dried preparation on maltodextrin together with the other ingredients. The results of the baking tests are shown in 30 Tables 8.1 to 8.4.

Table 8.1

Lipase LUS/kg flour	0	5,000	15,000	25,000
Specific volume of bread	4.43	4.43	4.22	4.37
Firmness Day 1	35	33	32	30
Firmness Day 7	90	90	85	73

Table 8.2

Lipase LUS/kg flour	0	5,000	15,000	25,000
Average diameter of the crumb pore, mm	2.96	2.33	2.47	2.65
Homogeneity of crumb pore, %	64.9	73.8	66.0	67.1
Porosity, %	85.9	84.7	85.5	85.1
Gluten index, %	42	45.5	55	65

5 Table 8.3

Lipase LUS/kg flour	0	5,000	15,000	25,000
Fatty acids, %	0.090	0.148	0.218	0.241
Monoglycerides, %	0.017	0.031	0.035	0.039
Diglycerides, %	0.020	0.036	0.040	0.045
Triglycerides, %	0.790	0.714	0.673	0.622

Table 8.4

Lipase LUS/kg flour	0	5,000	15,000	25,000
Monogalactosyl Diglyceride, %	0.073	0.040	0.025	0.018
Digalactosyl Diglyceride, %	0.244	0.220	0.182	0.127
Digalactosyl Monoglyceride, %	0.008	0.022	0.044	0.054
Phosphatidyl choline, %	0.064	0.073	0.055	0.041
Lysophosphatidyl choline, %	0.164	0.182	0.171	0.165

By the addition of up to about 5,000 LUS/kg flour of the lipase no change in bread volume was observed, but at a 5 higher dosage of lipase 3 there was a tendency to a small but not statistically significant decrease in volume (Table 8.1).

From the results in Table 8.2 it appears that lipase 3 improved the bread crumb homogeneity and that the average 10 diameter of the crumb pores was reduced significantly.

The gluten index also clearly correlated to the addition of lipase 3 as an indication of a more firm gluten caused by the modification of the wheat lipid components causing better dough stability and a more homogeneous bread pore structure. 15 However, these modifications appeared to be optimal at the addition of 5,000 LUS/kg flour of lipase 3 whereas a higher dosage resulted in a too strong modification of the wheat gluten.

20 The results of the GLC and HPLC analyses (Table 8.3) clearly demonstrated that the triglycerides in the dough were hydrolysed. But more interestingly, there was also observed a modification of the glycolipids, monogalactosyl diglyceride

and digalactosyl diglyceride. These components were converted to the more polar components monogalactosyl monoglyceride and digalactosyl monoglyceride. As digalactosyl monoglyceride is a more surface active component than digalactosyl diglyceride 5 it is assumed that this component contributed to the observed improved crumb cell structure and homogeneity. It also appeared that phospholipids like phosphatidyl choline were only modified to a very small extent.

10 8.3. Baking experiments with lipase 3 in Danish rolls

The effect of adding lipase 3 to a dough for making Danish rolls was evaluated. The enzyme was added as a freeze-dried preparation on maltodextrin together with the other ingredients. The results of the baking tests are shown in Tables 8.5 to 8.7.

Table 8.5

Lipase 3 LUS/kg flour	0	10,000	20,000	30,000
Specific volume of bread (45 min fermentation)	6.86	7.04	6.35	6.36
Specific volume of bread (65 min fermentation)	8.30	8.59	8.23	8.04
Subjective evaluation of crumb homogeneity	3	5	4	4

20

25

Table 8.6

Lipase 3 LUS/kg flour	0	10,000	20,000	30,000
Free fatty acids, %	0.060	0.126	0.173	0.211
Monoglycerides, %	0.028	0.050	0.054	0.063
Diglycerides, %	0.103	0.095	0.110	0.104
Triglycerides, %	0.705	0.561	0.472	0.436

Table 8.7

5

Lipase 3 LUS/kg flour	0	5,000	15,000	25,000
Digalactosyl Diglyceride, %	0.204	0.187	0.154	0.110
Digalactosyl Monoglyceride, %	0.007	0.026	0.047	0.074
Phosphatidyl choline, %	0.077	0.078	0.077	0.063
Lysophosphatidyl choline, %	0.153	0.161	0.162	0.150

It is apparent from the results shown in Table 8.5 that the addition of lipase 3 does not significantly increase the volume of the rolls. Furthermore, lipase 3 was found to 10 improve the homogeneity of the crumb.

The GLC and HPLC analyses of the wheat lipids, as shown in Tables 8.6 and 8.7, demonstrated the modification of these lipids.

EXAMPLE 9

Dough improving effect of glycerol oxidase and lipase

5 The effect of glycerol oxidase and lipase (separately or in combination) on dough strength was studied in a dough prepared according to the AACC Method 54-10. The dough was subjected to extensiograph measurements (Barbender Extensiograph EXEK/6) also according to AACC Method 54-10 with and 10 with out the addition of glycerol oxidase from *Aspergillus japonicus* combined with lipase from *Aspergillus oryzae* (GRIN-DAMYLTM EXEL 16, Bakery Enzyme, Danisco Ingredients). The dough with out addition of enzymes served as a control.

15 The principle of the above method is that the dough after forming is subjected to a load-extension test after resting at 30°C for 45, 90 and 135 minutes, respectively, using an extensiograph capable of recording a load-extension curve (extensiogram) which is an indication of the doughs resistance 20 to physical deformation when stretched. From this curve, the resistance to extension, B (height of curve) and the extensibility, C (total length of curve) can be calculated. The B/C ratio (D) is an indication of the baking strength of the flour dough. The results of the experiment are summarized 25 in Table 9.1 below.

Table 9.1: Extensigraph measurements of dough supplemented with glycerol oxidase and lipase

Sample (per kg flour)	Resting time (min)	B-value	C-value	D=B/C
Control	45	220	192	1.15
500 LUS lipase	45	225	190	1.18
1000 U glycerol oxidase	45	300	195	1.54
500 LUS lipase + 1000 U Glycerol oxidase	45	350	198	1.77
Control	90	240	196	1.22
500 LUS lipase	90	245	195	1.16
1000 U Glycerol oxidase	90	330	190	1.74
500 LUS lipase + 1000 U Glycerol oxidase	90	380	192	1.98
Control	135	260	188	1.38
500 LUS lipase	135	265	190	1.39
1000 U Glycerol oxidase	135	380	188	2.02
500 LUS lipase + 1000 U Glycerol oxidase	135	410	190	2.15

5 When the results from the above experiments are compared with regard to the differences between the control dough and the glycerol oxidase supplemented dough it appears that glycerol oxidase clearly has a strengthening effect. The B/C ratio was increased by 34%, 43% and 46% after 45, 90 and 135 minutes of 10 resting time respectively.

The addition of lipase only did not have any effect on the B/C ratio.

However, when supplementing the dough with a combination of glycerol oxidase and lipase, a further increase in the B/C ratio was seen as compared to bread prepared from dough 5 supplemented with glycerol oxidase only. The B/C ratio was increased by 54%, 62% and 56% after 45, 90 and 135 minutes respectively. This clearly indicates that the combined use of these two enzymes in the preparation of bread products has an enhancing effect on the baking strength.

10

EXAMPLE 10

Improvement of the specific volume of bread prepared from dough supplemented with glycerol oxidase and lipase

15

The effect of using glycerol oxidase and lipase (separately or in combination) on the specific bread volume and the crumb homogeneity was tested in a baking procedure for Danish rolls with a dough prepared as described in example 8. Glycerol 20 oxidase from *Aspergillus japonicus* and lipase 3 from *Aspergillus tubigensis* was added to the dough in different amounts. Dough without the addition of enzymes served as control. The fully proofed dough was baked at 220°C for 18 minutes with 12 seconds steam in a Bago-oven. After cooling 25 the rolls were weighed and the volume of the rolls were measured by the rape seed displacement method. The specific bread volume was determined as the volume of the bread (ml) divided by the weight of the bread (g). The crumb homogeneity was evaluated subjectively on a scale from 1 to 7, where 1 = 30 course inhomogeneous and 7 = nice homogeneous. The results from this experiment are summarized in Table 10.1 below.

Table 10.1: Specific volume and crumb homogeneity in bread supplemented with lipase and glycerol

Sample (per kg flour)	Specific vo- lume (ml/g)	Crumb homo- geneity
Control	5.45	1
1,000 U glycerol oxidase	6.75	2
10,000 LUS lipase	5.65	4
10,000 LUS lipase + 1,000 U glycerol oxidase	7.25	7

5

As can be seen in the above Table 10.1, the use of glycerol oxidase in the preparing of bread, significantly increased the bread volume (24%) as compared to bread prepared from a similar dough not supplemented with this enzyme. Addition of 10 U glycerol oxidase did not improve the crumb homogeneity significantly.

The use of lipase in the preparing of bread did not increase the specific volume of the bread, however a highly increased 15 pore homogeneity was observed.

The combined use of glycerol oxidase and lipase increased the specific volume of the bread with 33% as compared to bread prepared from a similar dough not supplemented with any of 20 the two enzymes.

In addition, the crumb homogeneity was highly improved by the combined use of lipase and glycerol oxidase as compared to the control bread and the breads prepared from dough supplemented with lipase and glycerol oxidase respectively.

This clearly indicates that the combination of lipase and glycerol oxidase in the preparation of bread has a synergis-

tic effect and significantly enhances the shape and appearance of the finished bread product.

EXAMPLE 11

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Hydrolysis of triglycerides and formation of glycerol in dough supplemented with lipase

In order to study the hydrolysis of triglycerides and the 10 formation of glycerol in a proofed dough supplemented with lipase, a dough for Danish rolls was prepared in the same manner as described in example 8. Different amounts of lipase (GRINDAMYL™ EXEL 16) was added to the dough, and the total lipid from the fully proofed dough was extracted and analyzed 15 by gas chromatography as described above.

Table 11.1: Triglycerides and glycerol in a dough as a function of lipase addition

Lipase addition (GRINDAMYL™ EXEL 16) (LUS per kg flour)	Glycerol (%)	Triglycerides (%)
0	2.2	7.88
500	2.2	6.22
1,250	2.4	5.99
2,500	2.8	5.37
3,750	2.9	5.47
5,000	3.0	5.55
7,500	3.1	5.03
10,000	3.0	4.39

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From the above experiment it is clear that the addition of

lipase to a dough has a hydrolyzing effect on the triglycerides present in the dough, which is seen as a decrease in the triglyceride content as function of the increased lipase addition. The resulting level of glycerol

5 increases as a function of the lipase addition.

These results suggests, that the improvement of the B/C ratio and the specific bread volume in bread prepared from dough supplemented with both glycerol oxidase and lipase, as was seen in example 9 and 10, could be due to that lipase addition to a dough is generating glycerol which further can act 10 as substrate for glycerol oxidase.

SUMMARY PARAGRAPHS

The present invention is defined in the claims and the accompanying description.

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For convenience other aspects of the present invention are presented herein by way of numbered paragraphs.

1. A method of improving the rheological properties of a
10 flour dough and the quality of the finished product made from
the dough, comprising adding to the dough 10 to 10,000 units
of a glycerol oxidase per kg of flour.

2. A method according to paragraph 1 wherein the glycerol
15 oxidase is derived from an organism selected from the group
consisting of a bacterial species, a fungal species, a yeast
species, an animal cell and a plant cell.

3. A method according to paragraph 2 wherein the fungal
20 species is selected from the group consisting of an
Aspergillus species, a *Neurospora* species and a *Penicillium*
species.

4. A method according to paragraph 1 wherein the resistance
25 to extension of the dough in terms of the ratio between
resistance to extension (height of curve, B) and the
extensibility (length of curve, C), i.e. the B/C ratio, as
measured by the AACC method 54-10 is increased by at least
10% relative to that of an otherwise similar dough not
30 containing glycerol oxidase.

5. A method according to paragraph 1 wherein the finished product is selected from the group consisting of a bread product, a noodle product and an alimentary paste product.

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6. A method according to paragraph 1 where at least one further enzyme is added to the dough ingredients, dough additives or the dough.

7. A method according to paragraph 6 wherein the further enzyme is selected from the group consisting of a cellulase, a hemicellulase, a starch degrading enzyme, an 5 oxidoreductase, a lipase and a protease.

8. A method of improving the rheological properties of a flour dough and the quality of the finished product made from the dough, comprising adding to the dough a glycerol oxidase 10 and a lipase.

9. A method according to paragraph 8 wherein the amount of glycerol oxidase is in the range of 10 to 10,000 units per kg flour.

15 10. A method according to paragraph 8 wherein the glycerol oxidase is derived from an organism selected from the group consisting of a bacterial species, a fungal species, a yeast species, an animal cell and a plant cell.

20 11. A method according to paragraph 10 wherein the fungal species is selected from the group consisting of an *Aspergillus* species, a *Neurospora* species and a *Penicillium* species.

25 12. A method according to paragraph 8 wherein the resistance to extension of the dough in terms of the ratio between resistance to extension (height of curve, B) and the extensibility (length of curve, C), i.e. the B/C ratio, as 30 measured by the AACC method 54-10 is increased by at least 10% relative to that of an otherwise similar dough not containing glycerol oxidase.

13. A method according to paragraph 8 wherein the finished 35 product is selected from the group consisting of a bread product, a noodle product and an alimentary paste product.

14. A method according to paragraph 8 where at least one

further enzyme is added to the dough ingredients, dough additives or the dough.

15. A method according to paragraph 14 wherein the further enzyme is selected from the group consisting of a cellulase, a hemicellulase, a starch degrading enzyme, an oxidoreductase, and a protease.

16. A method according to paragraph 8 wherein the amount of lipase is in the range of 10 to 100,000 LUS per kg of flour.

17. A method according to paragraph 8 wherein the lipase is derived from an organism selected from the group consisting of a bacterial species, a fungal species, a yeast species, an animal cell and a plant cell.

18. A method according to paragraph 17 wherein the lipase is derived from an *Aspergillus* species.

20 19. A method according to paragraph 18 wherein the *Aspergillus* species is selected from the group consisting of *A. tubigensis*, *A. oryzae* and *A. niger*.

20 20. A method according to paragraph 8 wherein at least 10% of the galactosyl diglycerides normally present in a flour dough is hydrolysed to the corresponding galactosyl monoglycerides.

21. A dough improving composition comprising a glycerol oxidase and at least one further dough ingredient or dough additive.

22. A composition according to paragraph 21 wherein the further dough additive is selected from the group consisting of a substrate for glycerol oxidase and a lipase.

35 23. A composition according to paragraph 22 which is a pre-mixture useful for preparing a baked product or in making a noodle product or an alimentary paste product.

24. A composition according to paragraph 21 which comprises an additive selected from the group consisting of an emulsifying agent and a hydrocolloid.

5 25. A composition according to paragraph 24 wherein the hydrocolloid is selected from the group consisting of an alginic acid, a carrageenan, a pectin and a vegetable gum.

10 26. A composition according to paragraph 21 wherein the amount of glycerol oxidase is in the range of 10 to 10,000 units per kg flour.

15 27. A composition according to paragraph 21 or 26, comprising as the further dough additive a lipase in an amount which is in the range of 10 to 100,000 LUS per kg flour.

20 28. Use of a glycerol oxidase for improving the rheological properties of a flour dough and the quality of the finished product made from the dough.

25 29. Use according to paragraph 28 wherein the improvement of the rheological properties include that the resistance to extension of the dough in terms of the ratio between resistance to extension (height of curve, B) and the extensibility (length of curve, C), i.e. the B/C ratio, as measured by the AACC method 54-10 is increased by at least 10% relative to that of an otherwise similar dough not containing glycerol oxidase.

30 30. Use of a glycerol oxidase and a lipase in combination for improving the rheological properties of a flour dough and the quality of the finished product made from the dough.

35 31. Use according to paragraph 30 wherein the improvement of the rheological properties of the dough include that the resistance to extension of the dough in terms of the ratio between resistance to extension (height of curve, B) and the

extensibility (length of curve, C), i.e. the B/C ratio, as measured by the AACC method 54-10 is increased by at least 10% relative to that of an otherwise similar dough that does not contain glycerol oxidase.

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32. Use according to paragraph 30 wherein the improvement of the quality of the finished product made from the dough is that the average pore diameter of the crumb of the bread made from the dough is reduced by at least 10%, relative to a 10 bread which is made from a bread dough without addition of the lipase.

33. Use according to paragraph 30 wherein the improvement of the quality of the finished product made from the dough is 15 that the pore homogeneity of the crumb of the bread made from the dough is increased by at least 5%, relative to a bread which is made from a bread dough without addition of the lipase.

20 34. Use according to paragraph 30 or 31 wherein the improvement of the rheological characteristics of the dough includes that the gluten index in the dough is increased by at least 5%, relative to a dough without addition of a 25 lipase, the gluten index is determined by means of a Glutomatic 2200 apparatus.

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>37</u> , line <u>6</u>	
B. IDENTIFICATION OF DEPOSIT <input type="checkbox"/> Further deposits are identified on an additional sheet	
Name of depositary institution The National Collections of Industrial and Marine Bacteria Limited (NCIMB)	
Address of depositary institution (including postal code and country) 23 St. Machar Drive, Aberdeen, Scotland United Kingdom, AB2 1RY	
Date of deposit 24 February 1997	Accession Number NCIMB 40863
C. ADDITIONAL INDICATIONS (leave blank if not applicable) <input type="checkbox"/> This information is continued on an additional sheet	
As regards the respective Patent Offices of the respective designated states, the applicants request that a sample of the deposited microorganisms only be made available to an expert nominated by the requester until the date on which the patent is granted or the date on which the application has been refused or withdrawn or is deemed to be withdrawn.	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable) The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g. 'Accession Number of Deposit')	
For receiving Office use only <input checked="" type="checkbox"/> This sheet was received with the international application	
For International Bureau use only <input type="checkbox"/> This sheet was received by the International Bureau on:	
Authorized officer  Jette Cordes Paulsen Head Clerk	
Authorized officer	